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14. ABSTRACT This 3 year multi-site project is now complete. It has successfully studied the role of toll like receptor 4 in both opioid and cocaine reward / reinforcement. In addition it has successfully documented the potential of (+)naltrexone (a clinically relevant, blood brain barrier permeable, <u>non</u> -opioid toll like receptor 4 antagonist) as an inhibitor of the rewarding / reinforcing effects of cocaine and opioids. Notably, it suppresses reinstatement to drug seeking (relapse) supportive of the interest in this compound for aiding drug abusers in the goal of avoiding relapse. In exploring brain mechanisms underlying these effects, we have accrued evidence supportive of both nucleus accumbens and ventral tegmental nucleus involvement.					
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1. Introduction:

Drug abuse and addiction is a globally pervasive issue, and has severely detrimental effects on society in terms of financial burden and undermining the health, well-being, and productivity of the addicted individual as well as their friends and family. Understandably, research for the past several decades has focused on neuronal targets for drug actions, as it is the mesolimbic dopamine pathway of the brain, which is comprised of dopaminergic neurons, that has been shown to underlie the rewarding and addictive effects of abused drugs. The resident immune cells of the brain, namely microglia and astrocytes, have recently garnered significant attention as it has become evident that they serve many roles in the brain and underlie many brain disorders due to their inflammatory signaling properties which have been shown to influence neuronal signaling and cell death. Here, we demonstrate that two drugs with high abuse liability, opioids and cocaine, activate proinflammatory signaling glial cells via interaction with Toll-Like Receptor 4 (TLR4), a receptor commonly expressed on microglial cells. We also show that activation of TLR4 signaling is necessary for drug-induced disruption of dopaminergic signaling leading to drug reward and abuse. Current treatments for opioid abuse are extremely limited in their effectiveness and sustainability whereas there is no approved pharmaceutical intervention for cocaine abuse. Our results indicate that targeting proinflammatory immune signaling in the brain via TLR4 may be the key to developing effective pharmaceutical therapies to aid in the treatment of addiction.

2. Keywords

Drug reward
Addiction
Opioid
Morphine
Heroin
Cocaine
Dopamine
Mesolimbic dopamine pathway
Conditioned Place Preference
Self-administration
Toll-Like Receptor 4
Microglia

3. Overall project summary

This 3 year multi-site project is now complete. It has successfully studied the role of toll like receptor 4 in both opioid and cocaine reward / reinforcement. In addition it has successfully documented the potential of (+)-naltrexone (a clinically relevant, blood brain barrier permeable, non-opioid toll like receptor 4 antagonist) as an inhibitor of the rewarding / reinforcing effects of cocaine and opioids. Notably, it suppresses reinstatement to drug seeking (relapse) supportive of the interest in this compound for aiding drug abusers in the goal of avoiding relapse. In exploring brain mechanisms underlying these effects, we have accrued evidence supportive of both nucleus accumbens and ventral tegmental nucleus involvement.

Background:

The reinforcing and addictive properties of abused drugs, such as morphine and cocaine, are largely attributed to their ability to activate the mesolimbic dopamine pathway, resulting in increased extracellular dopamine in the nucleus accumbens shell (NAc). Under normal circumstances, the ventral tegmental area (VTA) strictly regulates dopamine levels within the NAc. Morphine and cocaine are known to interact with the central

nervous system to produce distinctly different effects, both subjectively and physiologically; yet each drug is capable of increasing extracellular dopamine. To date, the bulk of research efforts have focused on how each drug interacts with its respective receptor targets on neurons. However, recently there has been more attention paid to the glial cells of the brain and how they might be involved in neurobiological mechanisms underlying the effects of drugs of abuse.

Morphine is known to act at mu-opioid receptors, which are located on neurons, both to produce its analgesic and rewarding/reinforcing effects. However, opioids—whether through prescription-based use to control pain or in abuse/illicit settings—have many unwanted side-effects, including tolerance (both for reward and pain relief), addiction, and severe withdrawal symptoms, among many others. Our laboratory recently published data demonstrating that morphine exerts many of these effects through activation of glial cells. Morphine-induced glial activation results in a powerful pro-inflammatory cascade, including the release of pro-inflammatory cytokines such as IL-1 β and TNF α . These cytokines, and other pro-inflammatory molecules are neuroexcitatory and have the ability to interact with and effect neuronal functioning. Furthermore, we identified that the receptor through which morphine was inducing glial activation is Toll-Like Receptor 4 (TLR4), an innate immune receptor responsible for detecting pathogens. After showing that blockade of the TLR4 receptor improved morphine's analgesic properties and attenuated analgesic tolerance, we began to investigate the role TLR4 signaling might have on morphine reinforcement. Preliminary studies demonstrated that systemic antagonism of TLR4 resulted in a blockade of both conditioned place preference (CPP) and self-administration, as well as a suppression of morphine-induced DA increase in the NAc. As intriguing as this finding is, it offers very little insight as to whether or not TLR4 signaling is directly involved in the mesolimbic pathway response to opioids, or whether there is some other less selective explanation for this phenomenon. Current pharmacological treatments for opioid addiction/abuse tend to be only effective and helping with decrease of illicit use, but require the continued use of a maintenance opioid, with lower abuse potential, that is costly and limited in success. Considering the increasing reports of opioid abuse, particularly abuse of prescription opioids, investigation into other treatment targets is of particular interest. TLR4 is an extremely interesting target to investigate, as early studies indicate that blockade of this receptor seems to preserve the desired effects of opioids (pain-relief) while diminishing unwanted effects (analgesic tolerance and reward/reinforcement leading to addiction/abuse).

Morphine is thought to exert most of its mesolimbic dopamine effects through actions in the VTA, where it disinhibits, or “turns down”, VTA control of dopaminergic projections, allowing for more dopamine release in the NAc. However, the prevailing hypothesis is that cocaine induces an increase of dopamine in the NAc through blockade of dopamine transporters (DAT), re-uptake and clearance of dopamine from the synapse, resulting in an increased concentration of dopamine. In particular, research has focused on cocaine blockade of DAT in the NAc. However, medication development approaches focusing on disrupting inhibition of DAT by cocaine are largely unsuccessful, and cocaine abuse remains widespread, highly problematic, and extremely difficult to treat. We have recently demonstrated that cocaine also interacts with the Toll-Like Receptor 4 (TLR4) complex and that this interaction may be an important contribution to the neurobiological effects of cocaine underlying reinforcement, leading to subsequent abuse and addiction. Systemic interruption of TLR4-cocaine signaling results in a blockade of models of drug reinforcement including cocaine-induced dopamine increases in the NAc, and a suppression of cocaine conditioned place preference, self-administration, and reinstatement to self-administration. These findings suggest that TLR4 signaling may be critical to both the reinforcing effects of cocaine and opioids such as morphine.

The purpose of this grant is to further investigate this remarkable finding to better understand the role of TLR4 signaling in drug reward/reinforcement in order to determine the potential clinical utility of this previously unknown mechanism. These results not only fundamentally alter and expand current understanding of the neurobiological mechanisms underlying drug reinforcement, but also offer a new potential target for medication development to treat cocaine abuse.

Task 1: Obtain approval from the Institutional Animal Care and Use Committee at University of Colorado Boulder for work to be done in the Watkins-Maier lab (University of Colorado-Boulder), Bachtell lab (University of Colorado-Boulder) and Katz lab (National Institute on Drug Abuse Intramural Research Program).

Task 1 has been completed on time for all sites and animal research is in progress.

Task 2: Receive (+)-naltrexone, as needed across the project period, from Dr. Kenner Rice (National Institute on Drug Abuse Intramural Research Program).

Task 2 is successfully undertaken; all (+)-naltrexone needed to date has been received by all research sites as committed by Dr. Kenner Rice

With accomplishment of Tasks 1 and 2, Milestone 1 was successfully achieved.

Watkins-Maier Research Lab:

Task 3 Aim 1A: Is morphine or cocaine CPP blocked by microinjecting a TLR4 antagonist (LPS-RS) into VTA or NAc shell?

Task 3 is complete in the Watkins-Maier lab.

We have evidence indicating that both cocaine and morphine interacts with and activates the TLR4-complex. Further, we have shown that cocaine interaction with TLR4 is an important modulator of drug-induced disruptions of the mesolimbic dopamine reward pathway that are thought to underlie the euphoric effects of drugs, leading to drug abuse and addiction (*see Task 4 for supporting data*). In order to investigate whether these neurochemical findings are relevant to drug reward behavior, we conducted CPP studies to test whether TLR4 antagonism within relevant brain regions of the mesolimbic dopamine pathway will correspondingly attenuate behavioral measures of drug reward. CPP is well established as an animal model of drug reward, correlating to the human experience of drug euphoria.

After identifying the ideal coordinates for bilateral VTA cannula placements, we established appropriate microinjection drug dosing, timing and techniques that would be physiologically and neurochemically relevant. We also established that this microinjection drug-dosing paradigm would not interfere with the CPP testing paradigm, which is considered to be a highly valid model of drug reward but is also well known to be sensitive to disruptions.

In the early stages of running the CPP microinjection experiments outlined in Task 3, the 500 year flood and 1000 year rain that took place in Colorado in September of 2013, damaged and destroyed many buildings in the Boulder area. Unfortunately, our CPP testing room and corresponding animal colony were in one of those areas that was seriously damaged by the flood. Not only were the animals currently undergoing CPP testing lost, but the rooms were so badly damaged that all animals and equipment had to be moved out immediately. The needed repairs were so extensive that early predictions of only 2-3 months were extended to more than 6

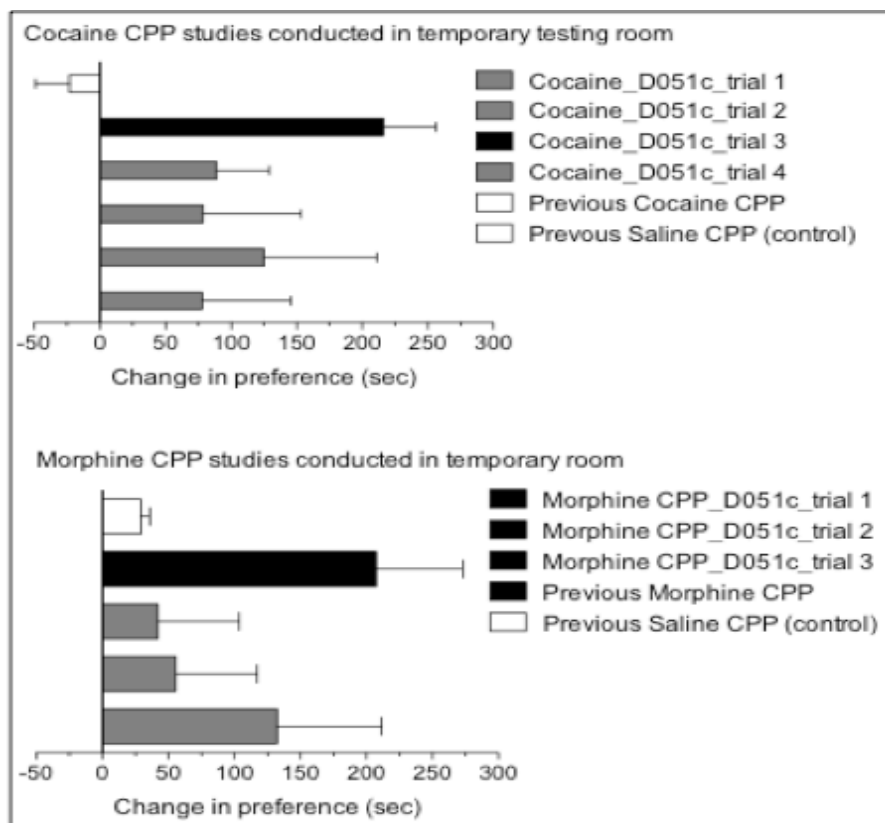


Figure 1: Following the extensive damage to our CPP testing suite and colony room due to the 500 year flood in 2013 that displaced us for more than 6 months, we attempted to move our CPP testing suite to a temporary room. The graphs demonstrate that neither (a) cocaine nor (b) morphine CPP could be established in the temporary testing room.

and other stimuli that might distract the rats or interfere with their conditioning/testing. We ran several series of experiments trying to re-establish our CPP effect with no success (fig. 1). Rats were unable to predictably learn to associate drug euphoria, from either cocaine (fig. 1a) or morphine (fig. 1b), with the correct corresponding compartment in the apparatus. We believe this is due to the comparative increase in activity (nearby testing, surgery, and wet lab rooms, increased traffic, equipment noise, etc.) in the area of the temporary testing space and due to ultrasonic vocalizations of rats in chronic pain experiments in nearby testing rooms (ultrasonic vocalizations readily pass through this building's walls). The failed CPP attempts in the temporary testing room, shown in figures 1 and 2, include data previously generated from our established CPP testing room for comparison.

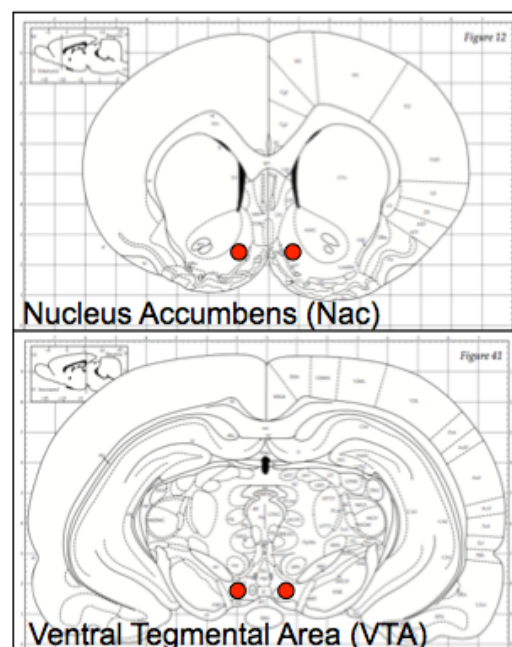


Figure 2: Depiction of the NAc and the VTA and the targets for implanted cannula used to microinject LPS-RS or saline vehicle. (*The Rat Brain in Stereotaxic Coordinates*, Paxinos and Watson, 2006).

months to conduct the repairs and bring all elements up to IACUC/OLAW code. In the meantime, we attempted to set up the CPP testing paradigm in a temporary space. CPP is an easily disrupted paradigm, as the testing room must be absolutely protected from noise, odors,

In July 2014 we received permission to move our CPP equipment back into our original testing space and to house animals in the adjoining colony room. We have since have been able to re-establish our CPP phenomenon. We have now completed all studies outlined in the aims of task 3.

Methods:

For both morphine and cocaine studies, rats had a microinjection cannula surgically implanted aiming at either the VTA or the NAc shell (fig. 2).

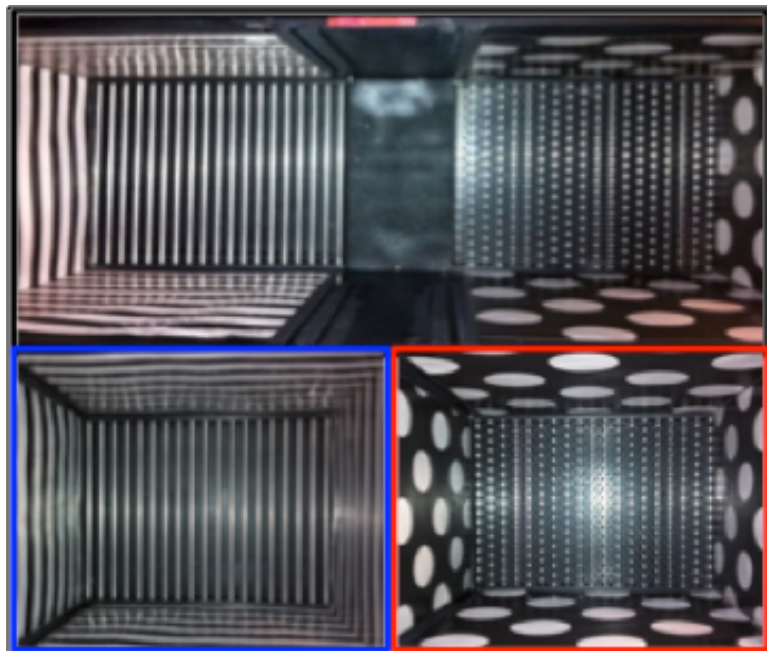


Figure 3: The CPP apparatus without the compartment dividers (top) as used during preexposure and place preference testing and with the walls inserted as used during conditioning sessions (bottom).

One week later, rats were pre-exposed to the CPP apparatus for 20 min in order to get a baseline measure of preference. Our CPP boxes are comprised of two distinct environments (one with black and white striped walls and metal bars for flooring and the other black with white polka dots with perforated metal plate for flooring (fig. 3). The amount of time rats spent on each side of the chamber is recorded with ANYmaze software receiving input from cameras mounted above each apparatus. Animals who spent less than 20% or more than 80% of the 20 min pre-exposure session one side of the apparatus were excluded from testing. For all studies reported here, only 2 animals were excluded due to biased pre-exposure score. Rats were then assigned to their treatment groups (see table 1) in a counter-balanced fashion. Animals received microinjections took place in the colony room, where each rat was wrapped in a towel and held gently, while a microinjector was inserted into the cannula. LPS-RS or saline was infused over a period of 1 minute and the microinjector was left in place for an additional 30 seconds. Rats were then transported to the

CPP testing room where they received systemic drug or saline injections approximately 10 minutes after their microinjection and were immediately placed in their assigned compartment of the CPP apparatus for conditioning. Conditioning studies lasted 28 minutes for cocaine studies and 40 minutes for morphine studies. Rats were then removed from the CPP apparatus and transported back to their home cages in the colony room. Conditioning sessions were conducted once daily, alternating between treatments for 6 days in cocaine studies and 8 days in morphine studies. The day following completion of all conditioning sessions, animals underwent Place Preference testing, conducted identically to Pre-Exposure sessions (fig. 4). Preference is determined by the subtracting the difference of time spent in Place Preference testing compared to Pre-Exposure

Treatment Groups	Cocaine studies (i.p. injections)	Morphine studies (s.c. injections)
Intra VTA microinjections	5 ug LPS-RS + Cocaine	5ug LPS-RS + Morphine
	5 ug LPS-RS + Saline	5ug LPS-RS + Saline
	Vehicle + Cocaine	Vehicle + Morphine
	Vehicle + Saline	Vehicle + Saline
Intra NAc microinjections	2.5 ug LPS-RS + Cocaine	2.5ug LPS-RS + Morphine
	2.5 ug LPS-RS + Saline	2.5ug LPS-RS + Saline
	Vehicle + Cocaine	Vehicle + Morphine
	Vehicle + Saline	Vehicle + Saline

Table 1: Treatments for all CPP and *in vivo* microdialysis studie

testing.

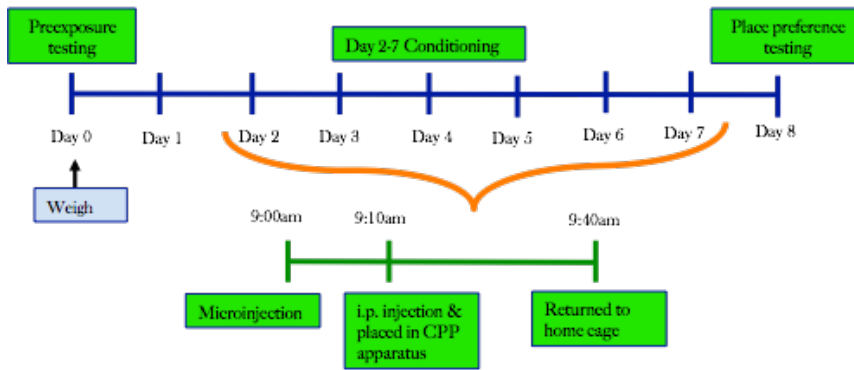


Figure 4: Timeline depicting testing and injection schedule for cocaine CPP studies. Morphine CPP studies were conducted in a similar fashion except that there were 8 days of conditioning which lasted 40 minutes each session.

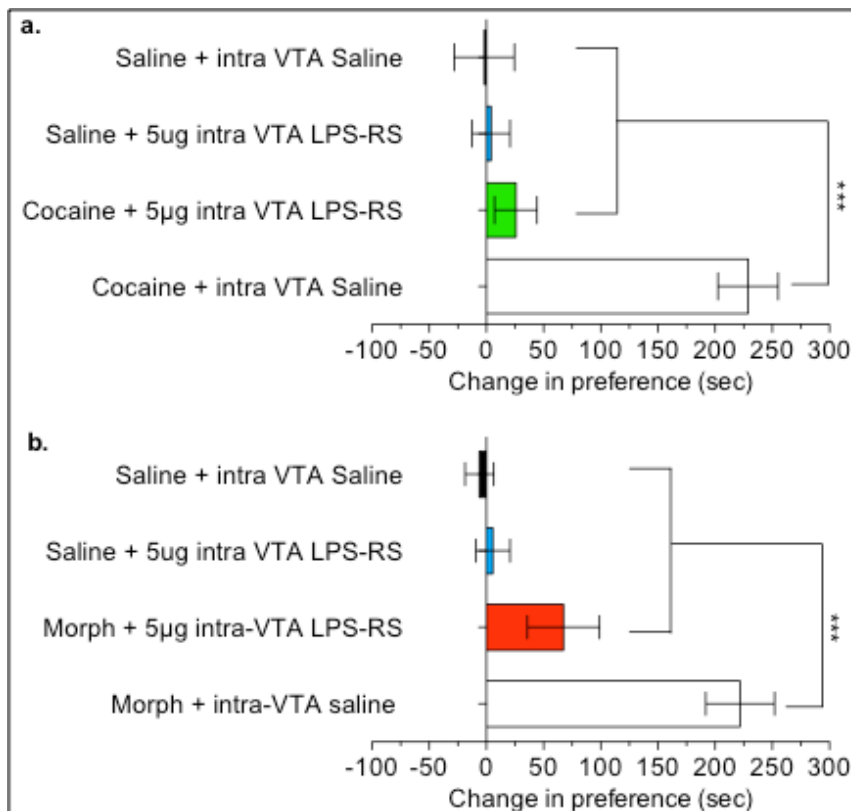


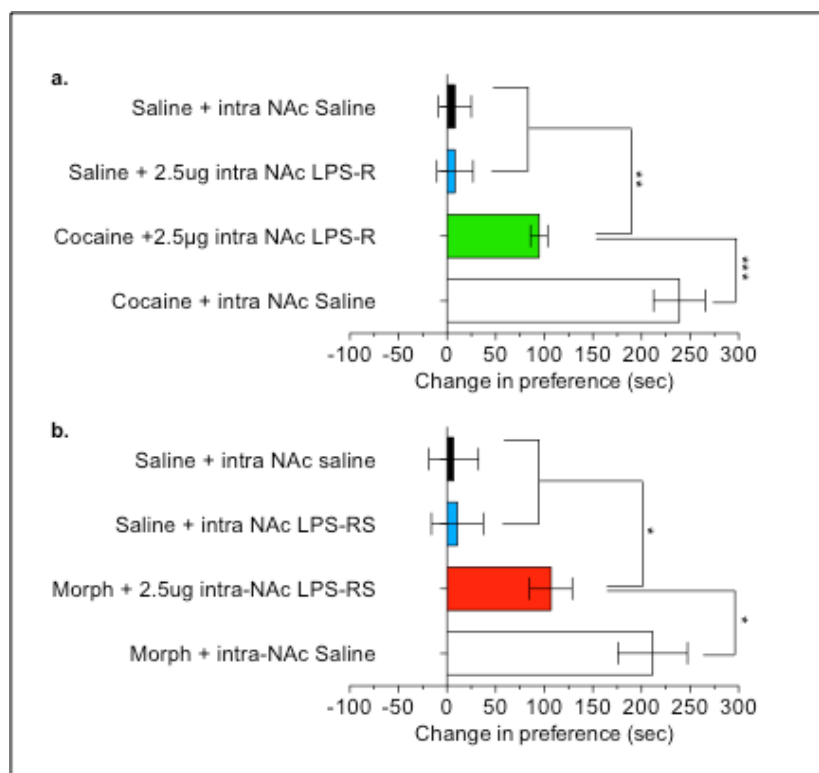
Figure 5: Intra-VTA infusion of the TLR4 antagonist LPS-RS resulted in a complete blockade of both (a) cocaine and (b) morphine CPP. Animals treated with LPS-RS alone had no change in preference, and were not different from their saline treated counterparts. Animals treated with either morphine or cocaine demonstrated a significantly higher preference for the drug-paired compartment (two-way ANOVA, bonferroni post-hoc, ***p < 0.001, n = 7-8 per group).

Results:

For both morphine and cocaine, intra VTA blockade of TLR4 signaling via microinjection of LPS-RS, *completely suppressed* drug-induced conditioned place preference (fig. 5). These findings are compelling for several reasons. The first is that the ability of cocaine or morphine to exert their rewarding effects are largely attributed to actions on neurons in the mesolimbic dopamine pathway. However, our data indicate that TLR4 activation is required in order for these drugs to produce behavioral measures of reward. This finding is very promising since, to date, there is very limited success in developing

treatments for opioid abuse and currently no approved pharmacotherapy for cocaine abuse. It may simply be that the scientific community had not yet uncovered the critical role of innate-immune signaling in the brain underlying drug reward and euphoria and thus have not targeted them for pharmacotherapeutic development. That the rewarding effects of morphine are dependent on the VTA is a commonly accepted explanation for its rewarding effects; however, it has long been thought that this was due to disinhibition of the VTA through its interactions with neuronal μ -opioid receptors. Our results indicating that morphine reward so heavily relies on innate immune signaling are novel; our finding that cocaine reward is heavily mediated by intra-VTA TLR4 signaling is both somewhat unconventional and remarkable. Classically, the rewarding effects of cocaine are attributed to its blockade of presynaptic DAT in the nucleus accumbens. Our data demonstrate that cocaine reward requires TLR4 signaling within the VTA in order to produce its rewarding effects.

Interestingly, antagonism of TLR4 signaling in the NAc shell also impacted drug reward, resulting in a significant attenuation of both morphine- and cocaine-induced place preference (fig. 6). Although our finding that TLR4 signaling in the NAc partially mediates cocaine reward is intriguing, as discussed previously, it is commonly accepted that cocaine reward is mediated through targets in the NAc. It was unexpected to find that the NAc also modulated morphine reward. These findings collectively indicate that drug-induced TLR4 signaling within the mesolimbic dopamine pathway is an important modulator of reward; particularly in the VTA, where our results indicate that TLR4 signaling is necessary for either cocaine or morphine to exert their rewarding effects. This discovery suggests that targeting immune activation initiated via TLR4 activation by cocaine and morphine may be a promising approach to developing a pharmacotherapeutic intervention to aid in the treatment of cocaine and morphine abuse.



Task 4 Aim 1B: Are cocaine-induced increases in extracellular DA in NAc shell blocked by microinjection of LPS-RS into the VTA or NAc shell?
Task 4 is complete in the Watkins-Maier lab.

The studies completed and discussed in task 3 demonstrate that cocaine and morphine activity at TLR4 within the mesolimbic dopamine pathway have important consequences for behavioral measures of drug reward. It is well established that drugs of abuse, such as morphine and cocaine, increase dopamine levels in the NAc and these increased dopamine levels correspond to the subjective experience of drug reward. While we have previously shown that systemic blockade of TLR4 signaling results in a complete suppression of morphine- and cocaine- induced increased of dopamine, it was unknown whether this results was a direct modulatory role of TLR4 in the VTA or NAc. We therefore conducted a series of *in vivo* microdialysis studies, which allow for the measurement of dopamine levels within the NAc in awake and mobile animals, to investigate whether TLR4 signaling within

Figure 6: Intra-VTA infusion of the TLR4 antagonist LPS-RS resulted attenuated both (a) cocaine and (b) morphine CPP. Animals treated with LPS-RS alone had no change in preference, and were not different from their saline treated counterparts. Animals treated with either morphine or cocaine demonstrated a significantly higher preference for the drug-paired compartment compared to all other groups (two-way ANOVA, bonferroni post-hoc, $*p < 0.05$, $n = 7-8$ per group).

the VTA or the NAc contributes to drug-induced disruptions of the mesolimbic dopamine pathway.

Methods:

For both morphine and cocaine studies, rats had one of two surgical procedures. For the studies to investigate TLR4 signaling within the VTA, a microinjection guide cannula was implanted aiming at the VTA and a microdialysis guide cannula was implanted in the NAc shell. One week later, rats were transported to the microdialysis testing room where there were temporarily housed overnight with bedding, and free choice food and water to habituate to the testing room. A microdialysis probe was inserted into the guide cannula to allow sampling of dopamine levels in the NAc, and attached with a tether. The next morning, after collecting three

baseline samples, animals received their drug treatments, identical to those used in Task 3 (table 1) and samples were collected for another three hours. Rats were then sacrificed and brains removed in order to verify cannula placements. For studies investigating TLR4 signaling within the NAc, a dual-microdialysis/microinjection guide cannula was implanted aiming at the NAc shell. The experiment was conducted as described above, except that a single dual probe/microinjector was inserted into the NAc. This specialized injector/probe allows for microinfusion of drug into the same region where dopamine measurements take place.

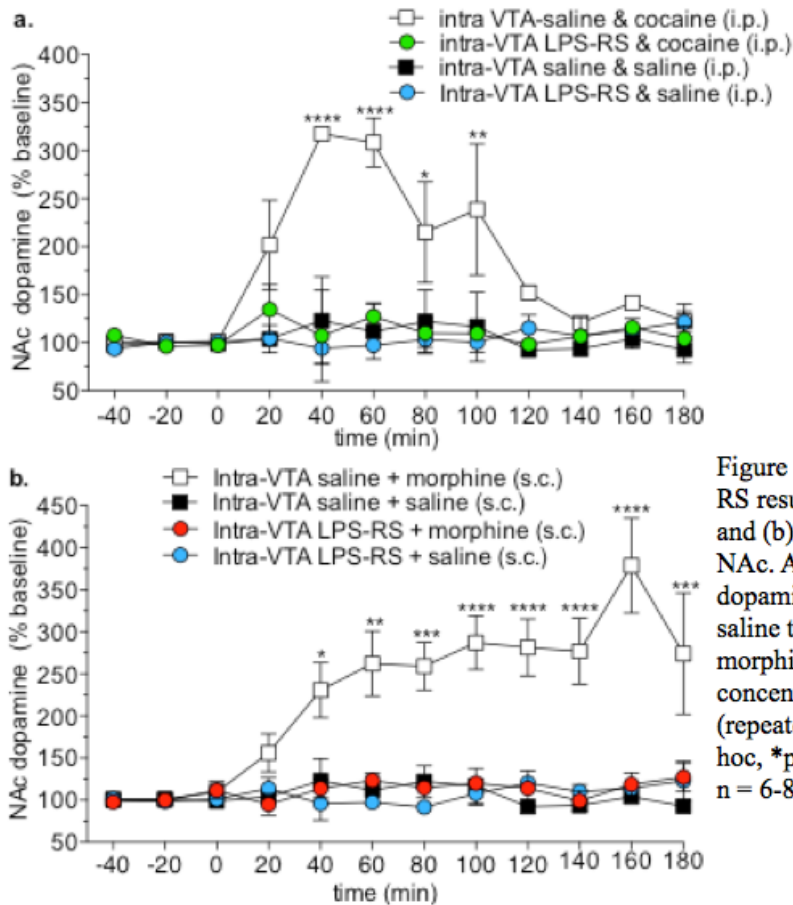


Figure 7: Intra-VTA infusion of the TLR4 antagonist LPS-RS resulted in a complete suppression of both (a) cocaine- and (b) morphine-induced dopamine increases within the NAc. Animals treated with LPS-RS alone had no change dopamine concentrations, and were not different from their saline treated counterparts. Animals treated with either morphine or cocaine demonstrated a significantly concentration of dopamine compared to all other groups (repeated measures two-way ANOVA, bonferroni post-hoc, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, $n = 6-8$ per group).

Results

Interestingly, we found that intra-VTA blockade of TLR4 signaling is particularly critical for morphine- or cocaine-induced increases of dopamine within the NAc. Intra-VTA microinjections of the TLR4 antagonist LPS-RS suppress both cocaine- and morphine-induced increases of dopamine within the NAc (fig. 7). These results are intriguing on many fronts. First is that they are somewhat expected in their potency. That there are other targets influencing regulations of the mesolimbic dopamine pathway is not an untested idea, but that a non-neuronal, immune cell target might have such potent modulatory power over this pathway is extremely novel and compelling. Because of the complete blockade of drug-induced dopamine increases observed with intra-VTA administration of LPS-RS, in order to ensure that intra-VTA LPS-RS administration wasn't just broadly "turning off" or interfering with mesolimbic dopamine pathway functioning, we conducted a control study. In this case, rats received intra VTA injections LPS-RS following by a microinjection of the endogenous peptide, neurotensin. Neurotensin has been shown to increase intra-NAc dopamine concentrations when injected into the VTA; given that neurotensin is endogenous in origin, it is very unlikely to interact with the TLR4 complex. Our results indicated that neurotensin-induced increases of NAc dopamine are preserved in the presence of intra-VTA LPS-RS and therefore it is unlikely that LPS-RS is ubiquitously inhibiting the mesolimbic dopamine pathway. The results of this control study further support that both cocaine and morphine bind to and activate TLR4 in the VTA which is required in order to produce increased dopamine concentrations in the NAc. As discussed in aim 3, morphine is well established to exert its effects in the VTA, albeit through a neuronal opioid-receptor target, so the importance of signaling within the VTA is characteristic of all opioids. However, although this finding is more exceptional in relation to cocaine's effects on the dopamine pathway, it

also may solve a long-standing question as to how cocaine triggers increased dopamine in the NAc. Although antagonistic actions on presynaptic dopamine transporters could lead to increased dopamine concentrations, it

has also been shown that cocaine disrupts the regular, pace-maker like firing of dopaminergic cells originating in the VTA. However, the mechanism underlying this effect is largely unknown. Our findings may suggest that cocaine increases dopamine cell firing through TLR4 signaling, as the subsequent release of proinflammatory molecules have neuroexcitatory actions on neurons.

Our studies investigating the effect of TLR4 signaling blockade within the NAc also have interesting results. Intra NAc administration of LPS-RS results in an attenuation of cocaine- and morphine- induced increases of dopamine within the NAc. (fig. 8). This suggests that TLR4 induced activation of innate immune signaling within the NAc may contribute to the ability of cocaine and morphine to disrupt dopamine signaling within the mesolimbic dopamine pathway.

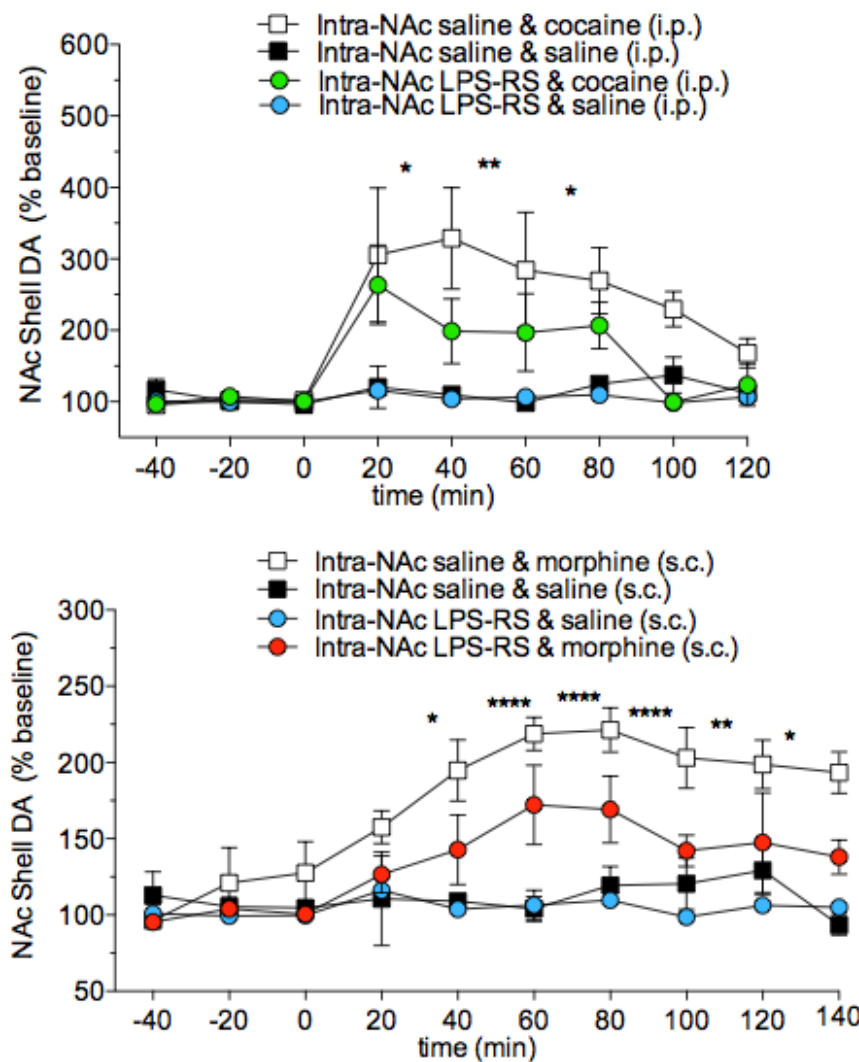


Figure 8: Intra-NAc infusion of the TLR4 antagonist LPS-RS attenuated both (a) cocaine- and (b) morphine-induced dopamine increases within the NAc. Animals treated with LPS-RS alone had no change dopamine concentrations, and were not different from their saline treated counterparts. Animals treated with either morphine or cocaine demonstrated a significantly concentration of dopamine compared to all other groups (repeated measures two-way ANOVA, bonferroni post-hoc, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, $n = 6-8$ per group).

Task 5 Aim 3A & 3B: Which cell type(s) (microglia, astrocytes, oligodendrocytes, endothelial cels, neurons) express TLR4 in VTA and/or NAc, basally vs. after chronic morphine/cocaine?

These experiments are nearly complete and final analysis/cell counts are underway. As discussed earlier, the 500 year flood in 2014 dramatically impacted our progress as our behavior testing rooms were severely damaged and an entire cohort of animals were lost, which set us behind schedule by approximately six months. Although this aim was proposed “if time and funds allow”, we still felt that it was important to investigate the questions that it would addressed in our final aim.

As discussed in our previous reports, through western blot screening, we have discovered that commercially available TLR4 antibodies are not selective; that is, they exhibit significant non-specific binding, which makes their use in immunohistochemistry unreliable. However, the TLR4 signaling complex is also comprised of an MD-2 co-factor, which is an integral component of cocaine- and morphine- induced TLR4 activation. We found an MD-2 antibody that western blots indicated was promising in its selectivity. We have since developed and fine-tuned the immunohistochemistry protocol to ensure clean staining in brain tissues with good signal to noise ratios.

Glial cells and the TLR4-MD2 complex are expressed throughout the brain, however, there is evidence that glial populations are heterozygous across brain regions. Given the importance of TLR4 signaling in the VTA, we examined whether there would be differences in MD-2 expression across brain regions of the mesolimbic dopamine reward pathway in naïve rat brains compared to the brains. Furthermore, although the TLR4-MD2 complex is known to be primarily located on microglial cells, it is unknown whether it might also be located on specialized populations of neurons. This would have an important impact on how to interpret our findings and in understanding how drugs of abuse exert their rewarding and addictive effects so that we can develop effective pharmacotherapeutic interventions. We have previously found that administering a microglial activator inhibitor, minocycline, reduces to morphine- and cocaine-induced CPP, which is supportive our findings and hypothesis that the TLR4-MD2 complex modulates drug reward through glial activation and immune signaling. However, minocycline is notoriously non-selective and at varying doses can have differing effects. In order to investigate what cell types express the TLR4-MD2 receptor complex, we conducted a fluorescent immunohistochemistry study. For this experiment we used antibodies to label MD2, microglial cells (OX-42), and dopamine cells (TH) of the VTA. We chose to investigate both naïve tissues, to understand what MD2 expression looks like at baseline, and brains collected from animals who were consistently self-administering cocaine, in order to assess any relevant changes. It is well known that repeated drug use leads to changes in the brain in an attempt to maintain homeostatic neural systems and that these changes underlie a wide-range of phenomenon underlying drug addiction. Glial cells are known to demonstrate priming or sensitization, where repeated activation leads to increasingly stronger and more potent signaling cascades. If glia are indeed becoming sensitized with repeated exposure to cocaine, they may upregulate expression or change morphology

that reflects their preparedness to shift from a quiescent state (where they are not releasing proinflammatory molecules) to an activated state. This IHC paradigm also allows us to address this question of priming or increased expression within the VTA after rats experience chronic cocaine exposure. All tissues have been collected and IHC processes and analysis are underway.

Results:

In the brains of naïve rats, we found that there is significantly more MD2 expression within the VTA compared to the NAc. This may shed some light our findings from task 3 and task 4, demonstrating that TLR4-MD2 signaling within the VTA is an integral aspect of morphine and cocaine signaling that leads to increased NAc dopamine and drug reward. (fig. 9)

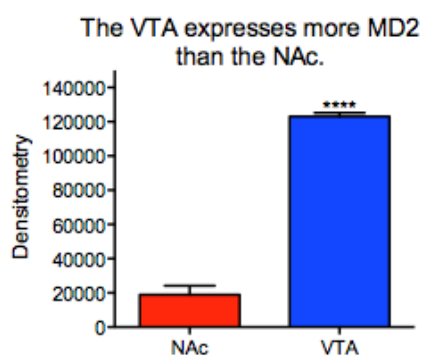
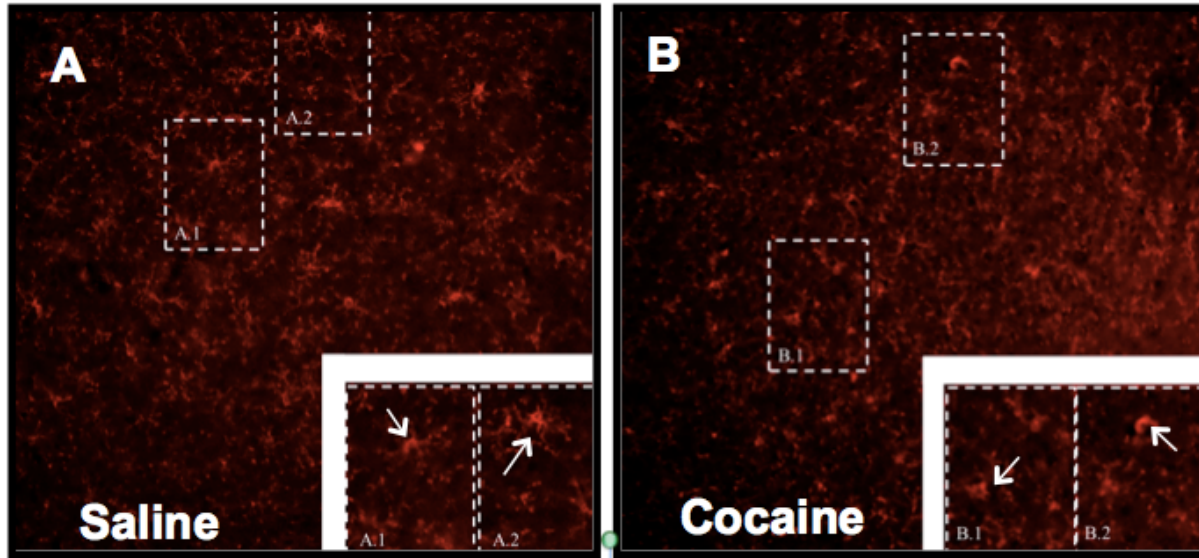


Figure 9: In naïve rats, the VTA expresses a higher concentration of MD2, the TLR4 signaling co-factor, compared to the NAc. Unpaired t-test, *** $p < 0.0001$, $n = 3$ per group.

Further, initial analysis of OX-42 microglial staining in the brains of rats who self-administered cocaine compared to rats who only self administered saline, suggests that there is both an increased expression of microglia and that these microglia display morphological changes (fig. 10). This change in morphology has important implications as it is reflective of what state the microglia are in. That is, quiescent microglia (i.e.



microglia at baseline functioning) demonstrate many processes and small cell bodies, to support their role in actively surveying their environment. The processes of activated microglia become amorphous as the cell mounts a proinflammatory response. Microglia that have been repeated

Figure 10: Fluorescent labeling of microglia in the VTA from animals chronically self-administering cocaine (b) compared to saline (a) one week following the final self-administration session. The differences in microglial morphology have interesting implications for the role of microglia following chronic cocaine administration. In panel a, note that microglia have small centers with many processes, as is expected of microglia in their baseline activity. In panel b, microglia become amorphous, no longer displaying processes, indicative of being in a proinflammatory or activated state. This characteristic is currently being quantified and further explored.

activated can become “primed”, that is, these microglia no longer resolve into a completely quiescent state and subsequent activation produces an exaggerated proinflammatory response. Given the data from CPP and *in vivo* microdialysis studies, there is the possibility the cocaine is causing microglia of the brain to become primed and over-reactive, which may contribute to or drive the dysfunction in dopaminergic signaling that occurs with repeated drug use and is known to underlie cocaine addiction. This characteristic of our IHC experiments is still in the process of quantification and analysis.

Optimization for the fluorescent triple labeling is currently underway. All primary and secondary antibodies have been purchased and tissues have been sliced and thaw mounted onto coated slides. This task is near completion, and data will be included in a manuscript that is currently in preparation.

Bachtell Research Lab:

Task 3 Aim 2A. Self- administration: Is cocaine reinforcement inhibited by systemic dosing with the TLR4 signaling inhibitor (+)-naltrexone? If so, is cocaine reinforcement inhibited by microinjecting LPS-RS into VTA or NAc shell?

Experiment 2A1: Acquisition of Cocaine Self-Administration

Methods: This experiment assessed the direct effect of TLR4 receptor antagonism on acquisition and maintenance of cocaine self-administration. We also tested the indirect effects of TLR4 antagonism on subsequent cue- and cocaine-induced reinstatement in the animals run to date. Osmotic minipumps (14-day

2mL) were filled with either (+)-naltrexone (15 mg/kg) or sterile water and implanted two days prior to the start of self-administration. Animals were then permitted to self-administered cocaine (0.5 mg/kg/infusion, iv) two hours per day over the next 17 days. After the self-administration session on day 13, the minipumps were removed and the animals continued with cocaine self-administration for an additional 4 days. The animals then underwent six days of extinction training followed by cue and then cocaine-induced reinstatement (15mg/kg, ip), where they were allowed to lever-press for two hours.

Results: Chronic administration of 15 mg/kg/day (+)-naltrexone revealed no change in the acquisition or maintenance of intravenous cocaine self-administration without prior lever-press training. Thus, (+)-naltrexone failed to affect the acquisition of self-administration during the first week of testing, and cocaine intake stabilized at similar levels in all groups during the second week of self-administration. After the last self-administration session, rats were progressed to extinction conditions to identify the indirect effects of (+)-naltrexone administration on cocaine seeking. Animals administered (+)-naltrexone during cocaine self-administration exhibited significant reduction in drug-paired lever responding compared to controls during the first extinction test. These data suggest that TLR4 inhibition during cocaine intake may decrease subsequent drug seeking that is indicative of drug craving. Following extinction, discrete cues that previously were paired with cocaine injections showed similar abilities to reinstate cocaine seeking in both (+)-naltrexone and control groups. Likewise, the administration of 15 mg/kg cocaine produced reinstatement to cocaine seeking similarly in both groups as well. Together, these data suggest that TLR4 inhibition with (+)-naltrexone administration during cocaine self-administration does not affect acquisition and maintenance of cocaine intake, but may reduce subsequent cocaine seeking. Studies in the upcoming year will assess whether increased doses of (+)-naltrexone effectively reduce the acquisition and maintenance of cocaine self-administration.

Experiment 2A2: Effects of (+)-Naltrexone on Cocaine Reinforcement

Methods: This experiment assessed the effects of TLR4 antagonisms on cocaine reinforcement using a progressive ratio schedule. The animals were allowed to self-administer cocaine (0.5 mg/kg/infusion, iv) for two hours per day on a fixed ratio 1 (FR1) schedule for six days, and were then moved to a fixed ratio 5 (FR5) schedule for four days. Osmotic minipumps (7-day 2mL) were filled with either (+)-naltrexone (15 mg/kg) or sterile water and implanted after the last day of self-administration on FR5 (one day prior to the start of the progressive ratio schedule). The animals self-administered cocaine on progressive ratio for five days, and the pumps were removed following the final self-administration session. The animals then underwent nine days of extinction training followed by two-hour cue and cocaine-induced reinstatement (15mg/kg, ip) sessions.

Results: The progressive ratio schedule of reinforcement is the hallmark procedure used to identify the reinforcing efficacy of drugs of abuse by assessing the amount of effort an animal is willing to exert to obtain cocaine reinforcement. Chronic administration of 15 mg/kg/day (+)-naltrexone during progressive ratio testing produced no change in either the number of cocaine infusions delivered or the final ratio completed (breakpoint) to earn a cocaine infusion. These findings suggest that 15 mg/kg/day (+)-naltrexone does not influence cocaine reinforcement mechanism. Future work will assess the effects of 30 mg/kg/day (+)-naltrexone on progressive ratio responding.

Task 4. Aim 2B. Is cocaine reinstatement to drug seeking blocked by systemic (+)-naltrexone? If so, is cocaine-induced reinstatement of drug seeking inhibited by LPS-RS microinjection into the VTA or NAc shell?

Experiment 2B1: Effects of Systemic (+)-Naltrexone on Cocaine reinstatement

Methods: This experiment assessed the effect of acute administration of (+)-naltrexone on cocaine-induced reinstatement. The animals self-administered cocaine (0.5 mg/kg/infusion, iv) for two hours per day for fifteen days. They then underwent extinction training for five days where lever presses were not reinforced. During reinstatement testing, the animals first had a two-hour extinction session immediately followed by a

pretreatment of two injections of (+)-naltrexone (0-15 mg/kg, sc) or saline vehicle spaced thirty minutes apart. After the second (+)-naltrexone injection, the animals received either a cocaine (15 mg/kg, ip) or saline vehicle prime. Non-reinforced lever pressing (active and inactive) was recorded during the two-hour session.

Results: All animals were trained to self-administer cocaine over 3 weeks. Lever responding was then extinguished in daily sessions where lever responding no longer produced the delivery of a cocaine infusion. After lever responding was extinguished to criterion, responding was reinstated by the administration of 15 mg/kg cocaine preceded by an acute (+)-naltrexone (7.5 or 15 mg/kg, ip) or vehicle injection. This study was analyzed with a 2-way ANOVA with cocaine prime (0 or 15 mg/kg) and (+)-naltrexone (dose) as the factors. The results of the analysis revealed significant main effects of Cocaine ($F_{1,50} = 36.34$, $p < 0.001$) and (+)-naltrexone ($F_{2,50} = 4.21$, $p < 0.02$) and a significant interaction ($F_{2,50} = 4.28$, $p < 0.02$). Further analyses of the interactive effects reveal significant cocaine seeking in vehicle-pretreated animals that was inhibited with a pretreatment of 15 mg/kg (+)-naltrexone (**Fig. 11**). These results suggest that systemic TLR4 antagonism with (+)-naltrexone is sufficient to inhibit cocaine seeking.

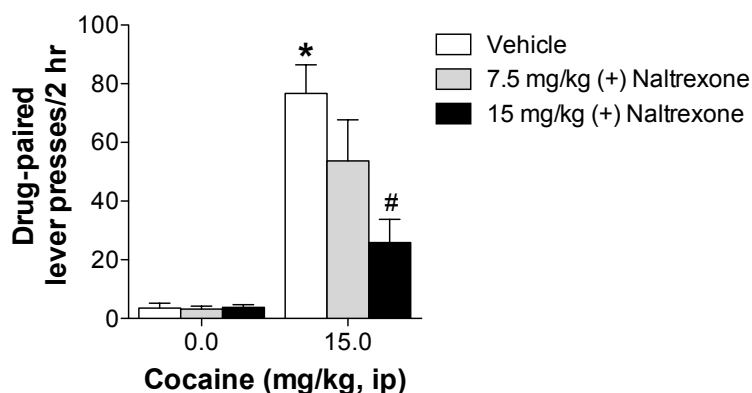


Figure 11. Acute administration of (+)-naltrexone dose-dependently reduced cocaine-primed reinstatement. * Significant from Vehicle/Vehicle, $p < 0.05$; # Significant from vehicle/15 mg/kg Cocaine, $p < 0.05$; $N = 10-12$ /group

Experiment 2B2: Effects of LPS-RS microinjections on Cocaine Reinstatement

Methods: These experiments are designed to assess the effect of TLR4 antagonism specifically in the nucleus accumbens shell (NAcSh) or ventral tegmental area (VTA) on cocaine-induced reinstatement. Prior to the start of self-administration, the animals were implanted with both an intravenous catheter and guide cannula directed into either the NAcSh or VTA. After recovery from surgery, the animals self-administered cocaine (0.5 mg/kg/infusion, iv) for two hours per day for fifteen days. Animals then underwent extinction training in daily two-hour extinction sessions for nine days. During reinstatement testing, the animals first had a two-hour extinction session immediately followed by a microinjection pre-treatment of LPS-RS (5 μ g/site) or saline vehicle followed by a cocaine (15 mg/kg, i.p.) or saline vehicle prime. Non-reinforced lever pressing (active and inactive) was recorded during the two-hour session.

Results: These experiments have been completed and the results are congruent with our hypotheses. Specifically, we have found that cocaine-induced reinstatement of lever presses was significantly blunted by site-specific infusions of the TLR4 antagonist, LPS-RS, into either the NAcSh or VTA (**Fig. 12**). The studies assessing LPS-RS effects in the NAcSh or VTA were analyzed with a 2-way ANOVA with lever and treatment as the factors. The results of the NAcSh study revealed significant main effects of lever ($F_{1,45} = 16.80$, $p < 0.002$) and treatment ($F_{2,45} = 12.43$, $p < 0.001$) and a significant interaction ($F_{2,45} = 8.43$, $p < 0.01$). Further analyses of the interactive effects reveal significant cocaine seeking in vehicle-pretreated animals that was inhibited with a pretreatment of LPS-RS (**Fig. 12**). Similarly, analysis of the VTA study revealed significant main effects of lever ($F_{1,53} = 80.88$, $p < 0.001$) and treatment ($F_{2,53} = 46.87$, $p < 0.001$) and a significant interaction ($F_{2,53} = 46.22$, $p < 0.001$). Analyses of the interactive effects also reveal significant cocaine seeking in vehicle-pretreated animals that was inhibited with a pretreatment of LPS-RS (**Fig. 12**). Together, these results

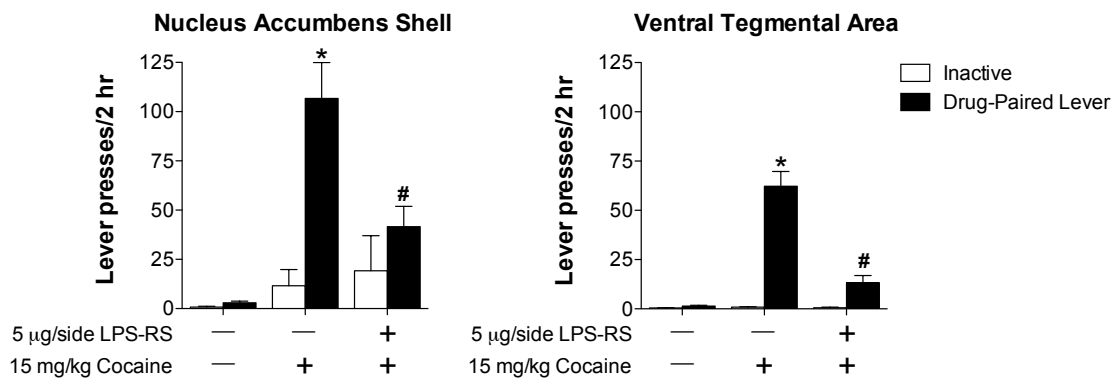


Figure 12. Administration of LPS-RS into either the nucleus accumbens shell or ventral tegmental area inhibited cocaine-primed reinstatement. * Significant reinstatement of lever pressing compared with Vehicle pretreated animals receiving a saline prime ($p < 0.05$). # Significant from vehicle-treated animals receiving 15 mg/kg cocaine prime ($p < 0.05$).

suggest that local inhibition of TLR4 in either the NAcSh or VTA is associated with cocaine relapse and is sufficient to inhibit cocaine seeking.

Katz Research Lab:

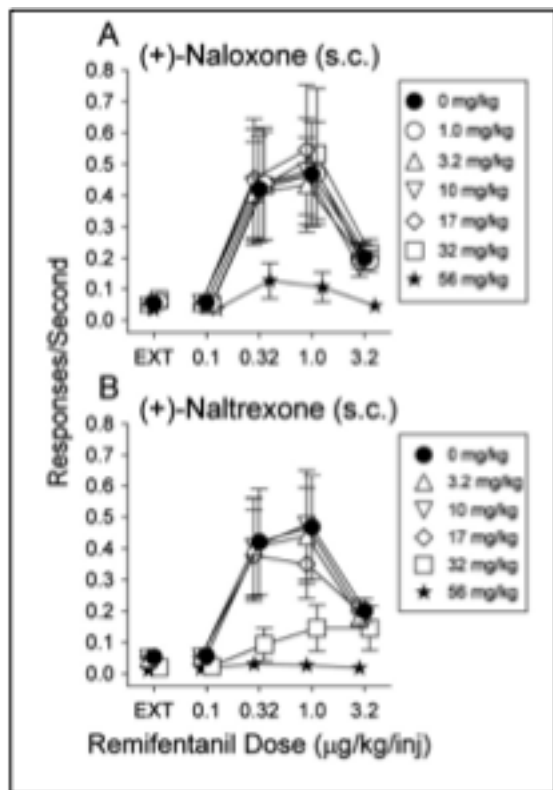
Dr. Takato Hiranita left our employ on 8/7/2013. He was replaced by Dr. Zachary Hurwitz who was with us from 7/29/13 to 3/1/2014. Dr. Hurwitz turned out to be a poor choice to replace Dr. Hiranita. During the short time that he was with us he learned the catheterization surgery and the fundamental techniques for training subjects to self-administer drugs. However just as we were prepared to initiate studies, Dr. Hurwitz left our employ for another position.

Dr. Claudio Zanettini joined us in late May of this year. We selected him from among about six final candidates because he had experience catheterizing subjects. Dr. Zanettini has been employed previously in well-known laboratories in the field of drug abuse and has the skills to successfully conduct and move the project forward (see CV in attachment). A detailed description of Dr. Zanettini activities since his start in May can be found in the Table below.

Dates		Activity
From	To	
5/19/2014	6/2/2014	Arrival and NIDA standard training for safe conduct of laboratory studies and animal care/welfare
6/3/2014	6/10/2014	Orientation to self-administration equipment in the NIDA labs, tested and modified self-administration program, ordered drugs.
6/10/2014	6/17/2014	Rat catheter implantation in jugular vein for self-administration
6/18/2014	6/29/2014	Post-surgery care of subjects, defined some technical details of the experiment
6/30/2014	7/7/2014	Rat self-administration of 0.1 mg/kg/inj heroin
7/8/2014	7/15/2014	Rat self-administration of 0.056 mg/kg/inj heroin
7/16/2014	8/6/2014	Extinction phase of the experiment and catheter implantation in 3 new subjects
8/7/2014	9/11/2014	Reinstatement phase of the experiment and catheter implantation in 4 new subjects
9/11/2014	9/21/2014	Rat self-administration of 0.1 mg/kg/inj heroin in newly implanted subjects and catheter implantation of 5 additional subjects
9/21/2014	Ongoing	Training for cannula implantations in VTA and NAc shell, infusions, and histological verification of placements with Dr. Gianluigi Tanda

Task 4. Aim 2A. Self-administration: Is morphine reinforcement inhibited by systemic dosing with the TLR4 signaling inhibitor (+)-naltrexone? If so, is morphine reinforcement inhibited by microinjecting LPS-RS into VTA or NAc shell?

The first part of this task (4b & 4c) has been completed and preliminary results were reported last year. Sprague-Dawley rats (Taconic Farms, Germantown, New York) weighing approximately 300 g at the start of the study, served as subjects. Subjects were acclimated to a temperature- and humidity-controlled vivarium for at least one week with a 12:12-h light:dark cycle (lights on at 07:00 hours) during which food (Scored Bacon Lover Treats, BIOSERV, Frenchtown, NJ) and tap water were available at all times. After acclimation, body weights were maintained at approximately 320 g by adjusting the daily food ration with water remaining available at all times in the home cages. Care of the subjects was in accordance with the guidelines of the National Institutes of Health and the National Institute on Drug Abuse Intramural Research Program Animal Care and Use Program, which is fully accredited by AAALAC International.



stimuli only). (+)-Naloxone and (+)-naltrexone were administered by the subcutaneous route at 5 min before sessions. Note that both drugs dose dependently decreased remifentanyl self-administration. Data are means \pm SEMs; $n = 6/\text{group}$.

Subjects were surgically prepared under anesthesia (ketamine 60.0 mg/kg, i.p. and xylazine 12.0 mg/kg, i.p.) with a chronic indwelling catheter in the right external jugular vein. The catheter exited the subject at the mid-scapular region of its back. Catheters were infused daily with 0.1 ml of a sterile saline solution containing heparin (30.0 IU/ml) and penicillin G potassium (250,000 IU/ml) to minimize the likelihood of infection and the formation of clots or fibroids. All animals were allowed to recover from surgery for approximately seven days before drug self-administration studies were initiated.

Experimental sessions were conducted daily with subjects placed in operant-conditioning chambers (modified ENV-203, Med Associates, St. Albans, VT) that measured 25.5 x 32.1 x 25.0 cm that were enclosed within sound-attenuating cubicles equipped with a fan for ventilation and white noise to mask extraneous sounds. On the front wall of each chamber were two response levers, 5.0 cm from the midline and 4.0 cm above the grid floor. A downward displacement of either lever with a force approximating 0.20 N defined a response, and always activated a relay mounted behind the front wall of the chamber producing an audible “feedback” click. Six light-emitting diodes (LEDs, three yellow and three green ones) were located in a row above each lever. A house light was located at 25 cm above the grid floor (near the ceiling) at the center of the front wall of the chamber. A receptacle for the delivery of 45-mg food pellets via a pellet dispenser (Med Associates, Model ENV-203-20), was mounted on the midline of the front wall between the two levers and 2.0 cm above the floor. A

syringe infusion pump (Model 22, Harvard Apparatus, Holliston, MA) placed above each chamber delivered injections of specified volumes from a 10 ml syringe. The syringe was connected by Tygon tubing to a single-channel fluid swivel (375 Series Single Channel Swivels, Plymouth Meeting, PA) which was mounted on a balance arm above the chamber.

Task 4b. Implant rats with indwelling jugular catheters, train on self-administration, pilot studies on (+)-naltrexone dose with dose adjustment as needed, test for effect of systemic (+)-naltrexone on morphine self-administration

Task 4c. Unblinding of data and data analysis

Rats were first trained on cocaine self-administration and were subsequently tested with remifentanyl substituted for cocaine. Remifentanyl injections reliably maintained self-administration at high rates that were dependent on dose of drug. **FIG 13** (filled circles) shows the inverted U-shaped dose-effect curve for remifentanyl; this shape of the dose-effect curve is characteristic of that for all drugs of abuse. Dose explorations (task 4b) indicated that the highest rate of responding was maintained at a remifentanyl dose of 1.0 $\mu\text{g/kg/inj}$, with lower response rates at higher and lower doses (**FIG 13**, filled circles). Response rates were significantly ($F(4,20) = 4.20$, $p = 0.013$) affected by remifentanyl dose, and post-hoc tests indicated that rates maintained by 1.0 $\mu\text{g/kg/inj}$ of remifentanyl were significantly greater than those obtained when responses had no consequences (EXT).

The effects of the TLR4 antagonists (+)-naloxone and (+)-naltrexone were tested on self-administration of the μ -opioid agonist, remifentanyl. Remifentanyl was chosen for testing rather than morphine due to its very short half-life which promotes high rates of self-administration and stability of lever pressing. The high rates of

responding maintained promote an exceptional signal-to-noise ratio. Rates of responding when responses have no consequences (EXT) are much lower than the rates of responding maintained by the 1.0 ug/kg dose of remifentanyl resulting in a S/N ratio for remifentanyl (0.001 mg/kg/inj: 0.689; EXT: 0.0145) of 47.6. This enhanced signal-to-noise ratio increases the sensitivity of the procedure for the detection of antagonism. In contrast, the S/N ratio for heroin (0.01 mg/kg/inj: 0.0727; EXT: 0.0129) is 5.64; which is about one order of magnitude lower.

The pilot studies with different doses of (+)-naloxone (s.c.) administered immediately before the self-administration sessions indicated a dose-dependent suppression of remifentanyl self-administration (**FIG 13A**). The maximally effective dose of (+)-naloxone was 56 mg/kg (compare filled circles to stars). A two-way repeated measures ANOVA indicated significant effects of remifentanyl dose ($F(4,40) = 5.22$, $p = 0.005$) and the interaction of remifentanyl and (+)-naloxone doses ($F(8,40) = 2.34$, $p = 0.036$). Post-hoc tests indicated that the effects of 56 mg/kg of (+)-naloxone significantly ($p \leq 0.012$) decreased response rates maintained by the 0.32 ($t = 2.99$) and 1.0 ($t = 2.98$) ug/kg/inj dose of remifentanyl. Decreases in remifentanyl self-administration were also obtained with i.p. injections of (+)-naloxone (data not shown).

The pilot studies with different doses of (+)-naltrexone (s.c.) immediately before the self-administration sessions also showed a dose-dependent suppression of remifentanyl self-administration (**FIG 13B**, compare filled circles to open squares and stars). Decreases in remifentanyl self-administration were obtained at a lower dose (32 mg/kg) of (+)-naltrexone compared with the effects of (+)-naloxone.

A second group of subjects was trained with food reinforcement in order to assess the specificity of the effects of (+)-naloxone on remifentanyl self-administration. Experimental procedures were identical to those detailed above except that each completion of five responses delivered a food pellet rather than an injection of remifentanyl. The selectivity of the effects of (+)-naloxone and (+)-naltrexone were assessed by comparing the effects at the maximal rates of responding maintained by either the dose of remifentanyl or the amount of food that maintained the highest rate of responding. As shown in **FIG 14**, the decreases in food reinforced responding occurred at about the same doses as those that decreased responding maintained by remifentanyl.

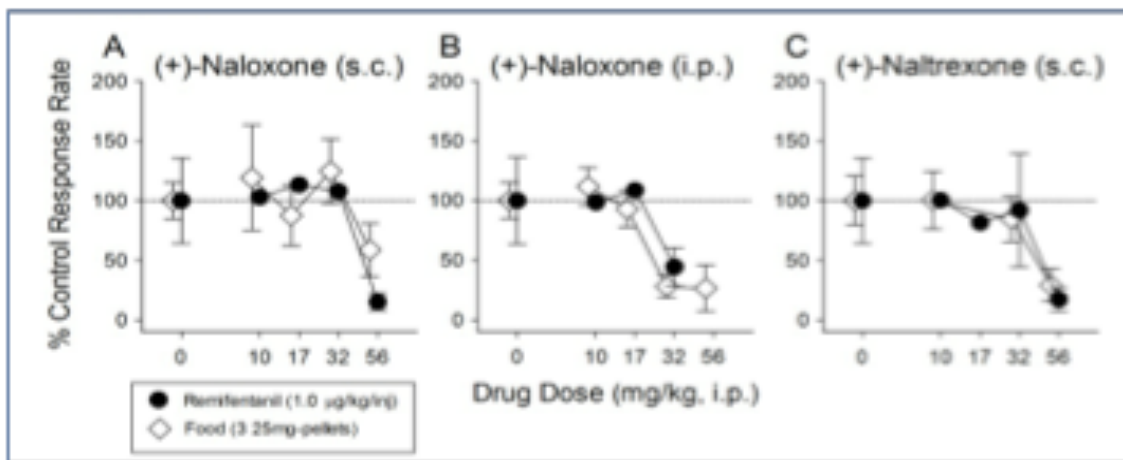


Figure 14: Effects of (+)-naloxone and (+)-naltrexone on responding maintained by injections of remifentanyl or food presentation. Ordinates: response rates as a % of control rates of responding; abscissae: drug dose in mg/kg. EXT: Extinction. (+)-Naloxone was administered by the subcutaneous or intraperitoneal route at 5 min before sessions. (+)-Naltrexone was administered by the subcutaneous route at 5 min before sessions. Note that (+)-naloxone and (+)-naltrexone were equipotent in decreasing responding maintained by injections of remifentanyl or food presentation. Data are means \pm SEMs; $n = 6$ /group.

Task 4d and 4e.
Cannula implantations in VTA and NAc shell, pilot studies to define doses, self-administration squads run, perfusion, histology to check cannula placements (after Aim 2B: task 5e), replacement of rats with poor cannula placements, site specificity studies as needed. Unblinding of data and data

analysis.

Dr. Zanettini is working with Dr. Gianluigi Tanda (NIDA/IRP) to learn all techniques necessary for these experiments. Dr. Zanettini has experience with these techniques, so the amount of time necessary for this training is not anticipated to be large. He will be learning all necessary techniques for cannula implantations in VTA and NAc shell, delivery of infusions at a rate sufficiently low that tissue damage will not result, and techniques for histological verification of cannula placements.

Task 5. Aim 2B. Is heroin reinstatement to drug seeking blocked by systemic (+)-naltrexone? If so, is morphine-induced reinstatement of drug seeking inhibited by LPS-RS microinjection into the VTA or NAc shell?

Sprague-Dawley rats (Taconic Farms, Germantown, New York) weighing approximately 300 g at the start of the study, served as subjects. Subjects were acclimated to a temperature- and humidity-controlled vivarium for at least one week with a 12:12-h light:dark cycle (lights on at 07:00 hours) during which food (Scored Bacon Lover Treats, BIOSERV, Frenchtown, NJ) and tap water were available at all times. After acclimation, body weights were maintained at approximately 320 g by adjusting the daily food ration. Water was available at all times in the home cages. Care of the subjects was in accordance with the guidelines of the National Institutes of Health and the National Institute on Drug Abuse Intramural Research Program Animal Care and Use Program, which is fully accredited by AAALAC International.

Subjects were surgically prepared under anesthesia (ketamine 60.0 mg/kg, i.p. and xylazine 12.0 mg/kg, i.p.) with a chronic indwelling catheter in the right external jugular vein. The catheter exited the subject at the mid-scapular region of its back. Catheters were infused daily with 0.1 ml of a sterile saline solution containing heparin (30.0 IU/ml) and penicillin G potassium (250,000 IU/ml) to minimize the likelihood of infection and the formation of clots or fibroids. All animals were allowed to recover from surgery for approximately seven days before drug self-administration studies were initiated.

Experimental sessions were conducted daily with subjects placed in operant-conditioning chambers (modified ENV-203, Med Associates, St. Albans, VT) that measured 25.5 x 32.1 x 25.0 cm that were enclosed within sound-attenuating cubicles equipped with a fan for ventilation and white noise to mask extraneous sounds. On the front wall of each chamber were two response levers, 5.0 cm from the midline and 4.0 cm above the grid floor. A downward displacement of either lever with a force approximating 0.20 N defined a response, and always activated a relay mounted behind the front wall of the chamber producing an audible “feedback” click. Six light-emitting diodes (LEDs, three yellow and three green ones) were located in a row above each lever. A house light was located at 25 cm above the grid floor (near the ceiling) at the center of the front wall of the chamber. A receptacle for the delivery of 45-mg food pellets via a pellet dispenser (Med Associates, Model ENV-203-20), was mounted on the midline of the front wall between the two levers and 2.0 cm above the floor. A syringe infusion pump (Model 22, Harvard Apparatus, Holliston, MA) placed above each chamber delivered injections of specified volumes from a 10 ml syringe. The syringe was connected by Tygon tubing to a single-channel fluid swivel (375 Series Single Channel Swivels, Plymouth Meeting, PA) which was mounted on a balance arm above the chamber.

Task 5b. Move rats from the systemic (+)-naltrexone study (Aim 2A, task 4b) into reinstatement paradigm, re-stabilize responding, drug withdrawal, test systemic (+)-naltrexone on drug cue (heroin)-induced drug seeking.

Task 5c. Unblinding of data and data analysis

It proved impractical to move subjects from task 4b into this study so experimentally naïve subjects were used for reinstatement paradigm and testing with systemic (+)-naltrexone on drug cue (morphine)-induced drug seeking.

Procedures for the acquisition of self-administration generally followed those described by Bossert et al. (2012). Experimental sessions were comprised of an initial 10-sec timeout period in which all lights were off and responding had no programmed consequences, followed by a responding period of 3 hours. In the responding period, one response on the active lever (FR 1) turned off the LED array above the lever, produced a click of a feedback relay and a 2.3 sec activation of the infusion pump during which the houselights were extinguished. Responding on the inactive lever produced a feedback clicker but no other programmed consequences.

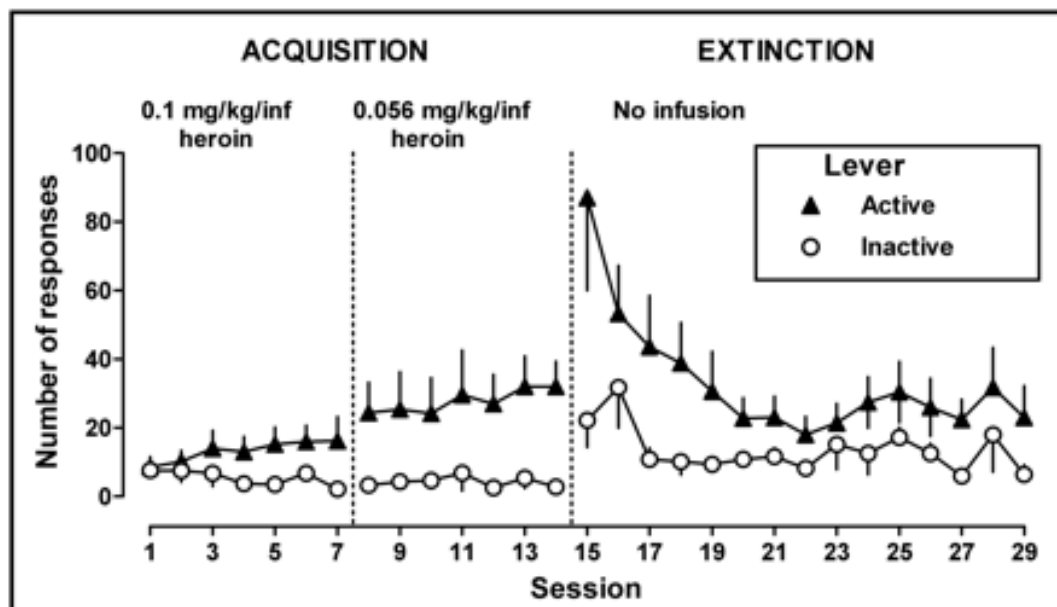


Figure 15. Acquisition and Extinction of heroin self-administration under the FR 1 schedule (Post-reinforcement Time-Out = 2.3 sec; session duration 3 hours). Ordinates: number of responses; Abscissae: sessions. Data are means \pm SEMs; N=5 subjects.

In Phases 1 (sessions 1-7) and 2 (sessions 8-14) of acquisition, each response produced, respectively, infusions of 0.1 and 0.056 mg/kg of heroin. Phase 3 (extinction) started on session 15 when the coefficient of variation for number of infusions during the last 3 sessions was less than 0.3. Phase 3 extinction sessions were identical to those in the preceding phases with the exception that no syringe was placed in the infusion pump and therefore responding on the active lever did not produce injections but did produce the stimuli previously paired with the delivery of heroin. Phase 3 extinction lasted 15 sessions.

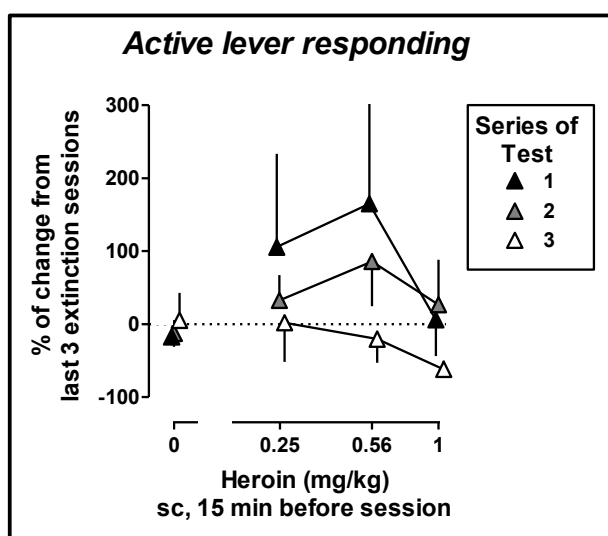


Figure 16. Heroin induced reinstatement of drug lever responding. Ordinates: percentage of change from last 3 extinction sessions. Abscissae: dose of heroin administered s.c 15 min before session. Data are mean \pm SEMs; N=5 subjects.

Numbers of responses on the active and inactive levers during acquisition and extinction are shown in **FIG 15**, and were consistent with previous published results (e.g. Bossert et al. 2012). During the first week (0.1 mg/kg/inj), responding on the active lever increased and reached levels above those on the inactive lever. Decreasing the unit dose to 0.056 mg/kg/inj increased active lever responses. At the end of Phase two the number of responses averaged 32 ± 7.3 responses per session (**FIG 15**). During extinction responding on both the active and inactive levers first increased and subsequently decreased to a plateau within about 6 sessions.

From session 30, subjects were tested for heroin-induced reinstatement of active lever responding. One of the aims of

this initial part of this task was to establish doses of heroin that would reliably induce reinstatement for the later assessment of (+)-naltrexone antagonism. In the first and second series of tests the dose of heroin that produced the maximal reinstatement was 0.56 mg/kg. The amount of reinstatement decreased with each series of tests (**FIG 16**). These results indicate that the dose that will be most reliable in producing reinstatement is 0.56 mg/kg and that the reinstating effectiveness of heroin is diminished with each test.

Aim 2B. Is heroin reinstatement to drug seeking blocked by systemic (+)-naltrexone? If so, is morphine-induced reinstatement of drug seeking inhibited by LPS-RS microinjection into the VTA or NAc shell?

Thirty animals underwent acquisition of heroin self-administration, extinction and three series of three reinstatement tests. Procedures were identical to the ones previously described with the exception that for 12 animals the unit dose of heroin in phase 1 and 2 was reduced by ¼ log unit. During reinstatement tests, animals were injected with saline or 15 mg/kg (+)-naltrexone (s.c, 15 min before session) and immediately after with of 0.56 mg/kg heroin or saline (s.c, 15 min before session) (Table below).

Reinstatement test	Heroin infusion dose during acquisition (mg/kg/inf)	
	0.1 (DAY1-7), 0.056 (DAY8-14)	0.056 (DAY1-7), 0.032 (DAY8-14)
(+) Naltrexone + heroin	N=6	N=6
saline + heroin	N=6	N=6
(+) Naltrexone + saline	N=6	

Task 5. Aim 2B. Number of animals that were trained in acquisition under the 2 different dosing conditions and the relative reinstatement tests.

In the group of animals trained in phase 1 and 2 with 0.1 and 0.056 mg/kg/inf heroin, respectively, the number of active lever responses during acquisition and extinction were comparable with the ones obtained in the previous experiment. In particular in the last day of acquisition the mean number of active responses was 27 ± 2 ; in the extinction phase animals reached a plateau of 20 ± 3 active lever responses (**FIG 17**).

Animals trained in phase 1 and 2 with 0.056 and 0.032 mg/kg/inf heroin, respectively, readily acquired self-administration with a progression similar to that for animals trained with larger doses of heroin (**FIG 17**). The numbers of responses with 0.032 mg/kg/inf were uniformly greater than those with 0.056 mg/kg/inf. In addition, the decay in rates of responding during extinction were greater in the subjects trained on the lower dose per injection.

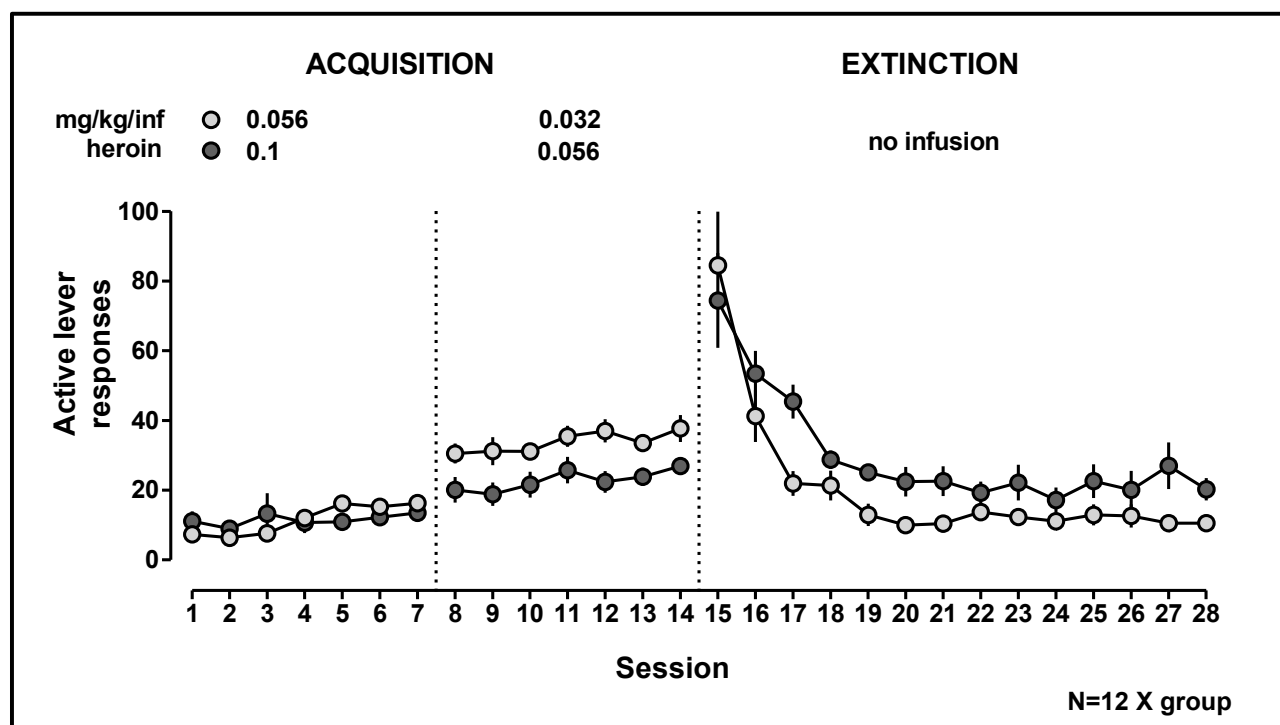


Figure 17. Acquisition and Extinction of heroin self-administration under FR 1 schedule. Ordinates: number of active lever responses. Abscissae: Number of sessions. Data are mean \pm SEMs; N=12 subjects per group.

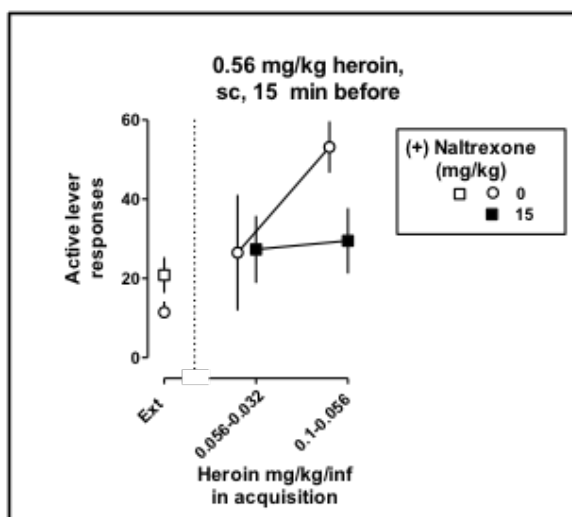


Figure 18. Effect of (+) naltrexone on active lever responses during extinction and heroin induced reinstatement as a function of the dose of heroin during acquisition. Ordinates: number of active lever responses. Abscissae: last day of extinction and unit doses of heroin received during acquisition. Data are mean \pm SEMs; N=6 subjects per group. Ext: N=12 per group (average all subjects in corresponding acquisition group)

Administration of a prime dose of heroin (0.56 mg/kg, s.c) reinstated active lever responding in the group of animals trained with larger doses of heroin during acquisition (0.1-0.056 mg/kg/inf) but not in the other group of animals (0.056-0.032 mg/kg/inf heroin) (open circles FIG 18). When (+)-naltrexone (15 mg/kg, sc) was administered in combination with the prime dose of heroin, it selectively decreased active lever responding only in the acquisition-dose condition (0.1-0.056 mg/kg/inf) in which heroin prime (+ saline) was effective in reinstating lever pressing (FIG 18 full square vs open circles). Administration of (+)-naltrexone and saline (acquisition condition 0.1-0.056 mg/kg/inf) did not reinstate active lever responding (data not shown).

Taken together, the current data are consistent with (+)-naltrexone decreasing the maximal effect in the heroin prime reinstatement dose-effect curve.

Fifteen animals were implanted with bilateral microinjection cannulas into the NAc shell (A:1.5 mm; L: \pm 1 mm; V: - 7 mm) and are undergoing acquisition of heroin self-administration (0.1-0.056 mg/kg/inf) and extinction as previously described. During reinstatement tests, heroin (0.56 mg/kg, s.c) or saline will be

administered before the session in combination with a NAc Shell microinjection of vehicle or LPS-RS (2.5 μ g in a volume of 0.5 μ l per side) (Table below).

Groups	Subjects
LPS-RS + heroin	5
LPS-RS + saline	5
saline + heroin	5

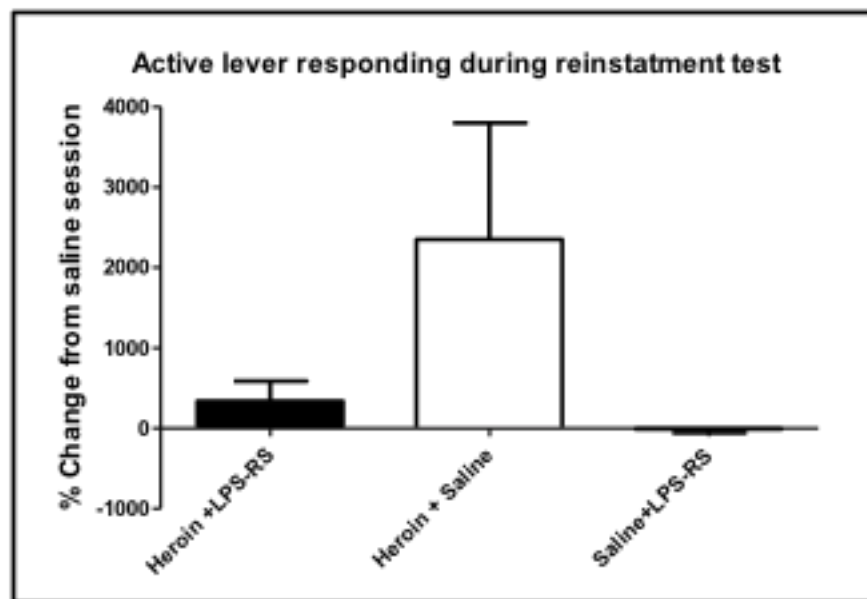
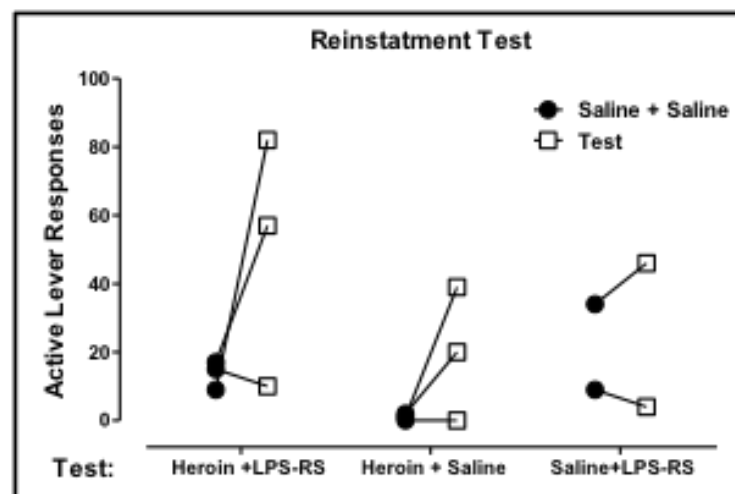


Figure 19. Effect of NAc Shell microinjections of LPS-RS (2.5µg per side) or saline on active lever responses during reinstatement tests in which saline or heroin was administered systemically 15 min before the session. Ordinates: % Change from session in which saline was administered systemically and into the NAc Shell. Group: Heroin+LPS-RS (N=3), Heroin+Saline (N=3), Saline+LPS-RS (N=2). Data are mean ± SEMs.

animals will undergo acquisition of heroin self-administration (0.1-0.056 mg/kg/inf), extinction and a reinstatement test in which heroin or saline will be administered before the session in combination with a VTA microinjection of vehicle or LPS-RS.

Figure 19. Individual subject performance after NAc Shell microinjections of LPS-RS (2.5µg per side) or saline during reinstatement tests in which saline or heroin was administered systemically 15 min before the session. Ordinates: Active lever responses. Group: Heroin+LPS-RS (N=3), Heroin+Saline (N=3), Saline+LPS-RS (N=2). Data are mean ± SEMs. Each line connects sessions obtained in the same animal. Full circles represent sessions in which saline was administered systemically and into the NAc Shell; open squares

ject contributing to the means in the previous graph is reported in **FIG 20**. Twelve



4. Key Research Accomplishments

- Several publications have been generated as a result of this work (listed below in part 6).
- The results of this work has been presented as part of multiple invited research seminars by the primary investigators and their trainees at conferences and universities. (listed below in part 6).
- This work was a significant part of Alexis Northcutt's PhD dissertation research project, successfully defended and her PhD awarded.

- This project supported the DoD's decision to fund Xalud Therapeutics to expedite development of (+)-naltrexone for treatment of drug abuse (and also neuropathic pain).

5. Conclusions

The research proposed for this grant has demonstrated that Toll-like receptor 4 (TLR4) is powerful modulator of drug abuse, as blocking TLR4 with (+)-naltrexone or (+)-naloxone suppresses multiple indices of drug reward and drug reinforcement for both opioids and cocaine. It is exciting that we are documenting the sites of action of TLR4 on drug abuse to the NAc shell and VTA, key structures in the rewarding and reinforcing effects of cocaine and opioids, the abused drugs under study. As (+)-naltrexone is in preclinical development aiming at human clinical trials, it is especially promising to have a blood brain barrier permeable, highly selective TLR4 antagonist that would be orally available, stable at room temperature, and appropriate for use from front lines through long-term use. As Xalud Therapeutics received \$2.7 million from the Army to move (+)-naltrexone toward FDA Investigational New Drug status, it will be ready for entry into human clinical trials near term. Given our ongoing results, this would be a spectacular step forward for treating warfighters and veterans alike for drug abuse indications.

6. Publications, abstracts, and presentations

Publications (additional manuscripts in preparation):

- Wang, X., Cochran, T.A., Hutchinson, M.R., Yin, H., and Watkins, L.R., Microglia and drug abuse, Microglia in Health and Disease, Springer, (2016) in press.
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- Grace, P.M., Maier, S.F., and Watkins, L.R., Opioid-induced central immune signaling: implications for opioid analgesia, Headache 55 (2015) 475-489.
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- Northcutt, A.L., Hutchinson, M.R., Wang, X., Barrata, M.V., Hiranita, T., Cochran, T.A., Pomrenze, M.B., Galer, E.L., Kopajtic, T.A., Li, C.M., Amat, J., Larson, G., Cooper, D.C., Huang, Y., O'Neill, C.E., Yin, H., Zahniser, N.R., Katz, J.L., Rice, K.C., Maier, S.F., Bachtell, R.K., Watkins, L.R., DAT isn't all that: cocaine reward requires toll like receptor 4 signaling, Molecular Psychiatry, (2015)
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7. Inventions, patents, and license

None

8. Reportable outcomes

Outcomes from the studies are summarized above. Follow-on grant proposals are in preparation for submission to (at least) NIH and to DoD as appropriate calls for proposals arise.

9. Other achievements

None

10. References

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11. Appendices

None